

Modulation of Biomarkers by Chemopreventive Agents in Smoke-exposed Rats¹

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ABSTRACT

Chemoprevention opens new perspectives in the prevention of cancer and other chronic degenerative diseases associated with tobacco smoking, exploitable in current smokers and, even more, in ex-smokers and passive smokers. Evaluation of biomarkers in animal models is an essential step for the preclinical assessment of efficacy and safety of potential chemopreventive agents. Groups of Sprague Dawley rats were exposed whole body to a mixture of sidestream and sidestream cigarette smoke for 22 consecutive days. Five chemopreventive agents were given either with drinking water (*N*-acetyl-L-cysteine, 1 g/kg body weight/day) or with the diet (1,2-dithiol-3-thione, 400 mg; Oltipraz, 400 mg; phenethyl isothiocyanate, 500 mg; and 5,6-benzoflavone, 100 mg/kg diet). The monitored biomarkers included: DNA adducts in bronchoalveolar lavage cells, tracheal epithelium, lung and heart; oxidative damage to pulmonary DNA; hemoglobin adducts of 4-aminobiphenyl and benzo[a]pyrene-7,8-diol-9,10-epoxide; micronucleated and polynucleated alveolar macrophages and micronucleated polychromatic erythrocytes in bone marrow. Exposure of rats to smoke resulted in dramatic alterations of all investigated parameters. *N*-Acetyl-L-cysteine, phenethyl isothiocyanate, and 5,6-benzoflavone exerted a significant protective effect on all alterations. 1,2-Dithiol-3-thione was a less effective inhibitor and exhibited both a systemic toxicity and genotoxicity in alveolar macrophages, whereas its substituted analogue Oltipraz showed limited protective effects in this model. Interestingly, combination of *N*-acetyl-L-cysteine with Oltipraz was the most potent treatment, resulting in an additive or more than additive inhibition of smoke-related DNA adducts in the lung and hemoglobin adducts. These results provide evidence for the differential ability of test agents to modulate smoke-related biomarkers in the respiratory tract and other body compartments and highlight the potential advantages in combining chemopreventive agents working with distinctive mechanisms.

INTRODUCTION

Worldwide tobacco smoke is the leading avoidable cause of cancer and other chronic degenerative diseases (1-3). Prevention of this plague is chiefly based on antismoke campaigns, which have been successful in attenuating the epidemic of lung cancer mortality in the male population of several countries (4, 5). A growing interest is centered on protection of the host organism either by means of dietary interventions or by using chemopreventive drugs (4). Chemoprevention may provide a complementary preventative approach in current smokers as well as an important strategy for lowering the risk in ex-smokers and in passive smokers.

Evaluation of biomarkers in animal models is an essential step for the preclinical assessment of efficacy and safety of potential chemopreventive agents (6). In previous studies, we investigated the optimal conditions needed for the time course formation and persistence of

Hb⁴ adducts⁵ oxidative damage to pulmonary DNA (7) and DNA adducts in organs (lung, heart, liver, bladder, and testis), tissues (dissected tracheal epithelium), and cells (BAL cells, mainly represented by PAM) of Sprague Dawley rats exposed whole body to ETS (7). ETS is a form of indoor air pollution resulting from the mixture of sidestream smoke and that portion of mainstream smoke that is released into ambient air by actively smoking individuals.

On the basis of these results, we designed a study aimed at evaluating the relative efficacy of several chemopreventive agents on a variety of smoke-related biomarkers in rats. Test agents included: (a) 1,2-D3T; (b) its substituted analogue OPZ, formerly used as a drug for schistosomiasis treatment; (c) PEITC, which is found at relatively high levels in watercress; (d) 5,6-BF, a synthetic flavonoid that interacts with the Ah receptor; and (e) NAC, an analogue and precursor of GSH. All agents were given p.o., either as dietary supplements (1,2-D3T, OPZ, PEITC, and 5,6-BF) or in drinking-water (NAC). A treatment regimen combining OPZ and NAC was also assayed. The monitored biomarkers included: DNA adducts in BAL cells, tracheal epithelium, lung, and heart; oxidative damage to pulmonary DNA, evaluated by measuring 8-OH-dG levels; Hb adducts of 4-ABP and BPDE; cytogenetic damage, assessed by measuring the frequency of MN and PN PAM and of MN PCE in bone marrow. The present studies provide evidence for the differential ability of various test agents to modulate ETS-related markers of biologically effective dose and early biological damage. Furthermore, the study with NAC and OPZ highlights the potential of combining chemopreventive agents with distinctive mechanisms of action.

MATERIALS AND METHODS

Animals. A total of 64 male Sprague Dawley rats, aged 8 weeks, were purchased from Harlan Italy (Correzzana, Milan, Italy). The animals were housed at the University of Genoa in a climatized environment at a temperature of 22 ± 1°C, relative humidity of 50 ± 5%, ventilation accounting for 15 air renewal cycles/h, and with a 12-h light-dark cycle. The rats were acclimatized for 7 days during which they were maintained on Teklad RM Rat/Mouse Diet (Harlan Italy) and were given drinking water *ad libitum*. Their weights at the time of exposure to ETS are reported in Table 1. Animal care was in accordance with Italian and institutional guidelines.

Exposure to Cigarette Smoke. The rats were divided into eight groups, each composed of eight animals. One group was kept in a filtered air environment (sham-exposed rats), whereas the remaining rats were exposed whole body to the smoke generated by Kentucky 2R1 reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY), having a declared content of 44.6 mg of total particulate matter and 2.45 mg of nicotine each. Before use, the cigarettes were kept for 48 h in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water.

A mixture of sidestream smoke (89%) and mainstream smoke (11%),

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⁴ The abbreviations used are: Hb, hemoglobin; BAL, bronchoalveolar lavage; PAM, pulmonary alveolar macrophages; ETS, environmental tobacco smoke; 1,2-D3T, 1,2-dithiol-3-thione; OPZ, Oltipraz or 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione; PEITC, phenethyl isothiocyanate; 5,6-BF, 5,6-benzoflavone; NAC, *N*-acetyl-L-cysteine; GSH, reduced glutathione; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; 4-ABP, 4-aminobiphenyl; 4'-F-ABP, 4'-fluoroaminobiphenyl; BPDE, benzo[a]pyrene-7,8-diol-9,10-epoxide; MN, micronucleated; PN, polynucleated; PCE, polychromatic erythrocytes; DRZ, diagonal radioactive zone; GC-MS, gas chromatography-mass spectroscopy.

⁵ S. R. Myers et al., manuscript in preparation.

Table 1 Body weight gain of rats within each of the experimental groups

Day of the experiment	Body wt (g)							
	Sham	ETS	ETS + OPZ	ETS + 1,2-D3T	ETS + NAC	ETS + NAC + OPZ	ETS + PEITC	ETS + 5,6-BF
0	295.9 ± 7.0 ^a	300.0 ± 9.0	302.1 ± 10.6	299.2 ± 8.4	305.5 ± 8.7	303.6 ± 6.5	305.1 ± 8.5	304.2 ± 10.9
7	303.9 ± 5.8	308.6 ± 4.9	303.1 ± 6.7	286.1 ± 11.1 ^{b,c}	312.3 ± 10.4	297.3 ± 8.2	305.0 ± 8.4	311.6 ± 10.1
14	335.0 ± 7.4	327.1 ± 14.0	324.0 ± 9.3 ^d	293.6 ± 8.6 ^{b,c}	329.1 ± 7.8	325.9 ± 14.8	321.3 ± 9.3 ^e	329.6 ± 14.7 ^d
21	347.9 ± 10.1	332.0 ± 15.5 ^d	329.8 ± 11.7 ^e	292.5 ± 10.8 ^{b,c}	332.4 ± 9.8 ^e	334.3 ± 13.2 ^d	322.6 ± 11.6 ^e	329.3 ± 14.7 ^d
28	366.3 ± 11.3	338.4 ± 18.4 ^e	337.3 ± 11.4 ^b	293.5 ± 11.5 ^{b,c}	330.0 ± 12.6 ^b	331.8 ± 9.8 ^b	327.4 ± 9.7 ^b	334.6 ± 12.8 ^b

^a Mean ± SD.^b $P < 0.05$, ^c $P < 0.01$, and ^d $P < 0.001$, compared with sham-exposed rats.^e $P < 0.001$, compared with ETS-exposed rats.

mimicking an exposure to ETS, was produced by using a smoking machine (model TE-10; Teague Enterprises, Davis, CA), where each smoldering cigarette was puffed for 2 s, once every min for a total of eight puffs, at a flow rate of 1.05 liters/min to provide a standard puff of 35 cm³ (8). The machine was adjusted to burn five cigarettes at one time, 6 h a day divided in two 3-h rounds with a 3-h interval, for 28 consecutive days. Sidestream and mainstream smokes were aspirated in a mixing chamber before distribution to four exposure chambers. Under those conditions, the total particulate matter in the exposure chambers was on an average 83 mg/m³, and CO concentration was 350 ppm. The position of the cages in the exposure chambers was rotated daily.

Treatment with Chemopreventive Agents. Of the seven groups of ETS-exposed rats, one was kept as a positive control, without any further treatment. The remaining rats received chemopreventive agents, starting 3 days before the first day of exposure to ETS and continuing until the end of the experiment.

5,6-BF, purchased from Sigma Chemical Co. (St. Louis, MO), and OPZ, 1,2-D3T, and PEITC, supplied by the Division of Cancer Prevention Repository, National Cancer Institute (Rockville, MD), were incorporated in Teklad diet (Harlan Teklad, Madison, WI) at concentrations of either 400 mg/kg diet (OPZ and 1,2-D3T) or 500 mg/kg diet (PEITC and 5,6-BF). The diets were prepared at the University of Alabama at Birmingham by adding the appropriate amount of chemopreventive agent to 8.0 kg of Teklad (4%) mash diet, using a Patterson-Kelly twin-shell blender with intensifier bar. The diets were mixed for 20 min, frozen at -80°C, and shipped to the University of Genoa, where they were kept at 4°C until use. As evaluated in preliminary HPLC analyses performed at the University of Alabama at Birmingham, the chemopreventive agents were stable in the diet for at least 28 days. NAC, either individually or combined with dietary OPZ, was used in the form of a commercial product (Fluimucil; Zambon, Vicenza, Italy), which was dissolved daily in drinking water at a concentration accounting for a calculated intake of ~1000 mg/kg body weight/day. The actual intake of each chemopreventive agent, in mg/kg body weight/day, was calculated by taking into account food and water consumption and the body weight gain of rats (see "Results" and Table 2).

Collection of Biological Samples. After the 28-day period of exposure to ETS, the animals were starved for 24 h, anesthetized with ethyl ether, and killed by cervical dislocation. The blood was immediately drawn by heart puncture and fractionated into hematocrit and plasma, which were stored separately at -80°C. Packaged RBCs were shipped in dry ice to the University of Louisville for the analysis of Hb adducts. BAL was performed by lavaging the lungs of each rat with two 10-ml aliquots of cold (4°C) 0.15 M NaCl infused via a cannula inserted into the trachea. A portion of BAL cells from each

animal was washed twice with RPMI 1640 and then spun onto slides in a cyto centrifuge and fixed with methanol for cytogenetic analyses. The remaining portions of BAL cells, after washing, were pooled within each experimental group of eight rats. The left femur of each rat was removed and dissected, and bone marrow cells were collected, smeared on slides, and air-dried for cytogenetic analyses. The trachea, lungs, and heart were collected. The ciliated epithelium of the trachea was accurately scraped. All cells, tissues, and organs were stored at -80°C until use.

DNA Purification. Heart, lungs, and tracheal epithelium from each rat and BAL cells pooled within each group of rats were thawed and homogenized in a Potter-Elvehjem apparatus at 4°C in 250 mM sucrose, 5 mM DTT, 50 mM Tris-HCl (pH 7.6). DNA was isolated by solvent extraction using an automatic extractor (Genepure 341; Applied Biosystems, Foster City, CA) according to the method of Gupta (9), with some minor modifications as described previously (10). Due to the low amount of material available for each sample, DNA extraction from tracheal epithelium and BAL cells was achieved by using the same procedure, except that 30 µl of Quik-Precip (Edge-Biosystems, Gaithersburg, MD) were added during precipitation with alcohol. DNA was eluted with water. Purity of DNA was checked by spectrophotometric analysis (10).

Detection of DNA Adducts. Aliquots of 6 µg of DNA were assayed for the presence of DNA adducts by butanol extraction, as described previously (11). Each sample was labeled with 64 µCi of carrier-free [³²P]ATP (ICN Biochemicals, Irvine, CA), with a specific activity of ≥7000 Ci/mmol. TLC was carried out on sheets of polyethyleneimine (Macherey and Nagel, Düren, Germany) according to standard procedures (11). Based on the results of a previous study (7), 7 M urea and 3 M lithium formate (System A) were used in D3. However, in some experiments (see "Results"), we comparatively used isopropanol:4 M ammonium hydroxide (1:1, v/v; System B). Autoradiography was performed by using a ³²P InstantImager Electronic Autoradiographic System equipped with InstantQuant software (model A2024; Packard, Meriden, CT). The relative adduct labeling index (cpm adduct/cpm normal nucleotides) was calculated, and DNA adduct levels in each sample were expressed as DNA adducts/10⁸ nucleotides. Due to the different amounts of available DNA, the samples were assayed either in two separate experiments (individual tracheal epithelia and pooled BAL cells) or three separate experiments (individual hearts and lungs). A 7R,8S,9S-trihydroxy-10R-(N²-deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo(a)pyrene reference standard (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) was used as a positive quality control to check the labeling efficiency in each experiment. On the whole, 528 ³²P postlabeling analyses were performed.

Table 2 Average intake of chemopreventive agents during the 4 weeks of exposure to ETS

Wks of exposure to ETS	Unit	Intake of chemopreventive agent (mg or mmol/day/kg body wt)					
		OPZ ^a	1,2-D3T ^a	NAC ^b	OPZ ^c + NAC ^b	PEITC ^c	5,6-BF ^c
1	mg	23.6	21.4	103	24.5 + 1014	31.3	30.3
	mmol	0.1	0.2	6.9	0.1 + 6.2	0.2	0.1
2	mg	25.2	26.0	106	24.8 + 1118	31.0	30.5
	mmol	0.1	0.2	6.7	0.1 + 6.9	0.2	0.1
3	mg	23.2	26.1	133	19.3 + 1333	31.1	29.4
	mmol	0.1	0.2	8.2	0.1 + 6.2	0.2	0.1
4	mg	20.5	25.9	131	19.3 + 1298	30.8	27.6
	mmol	0.1	0.2	8.0	0.1 + 6.0	0.2	0.1

^a 400 ppm with the diet.^b 10,000 ppm with drinking water.^c 500 ppm with the diet.

Detection of Oxidative DNA Damage. Oxidative damage was evaluated in lung DNA samples pooled within each experimental group. A ^{32}P postlabeling procedure was used, as previously described (7, 12). As previously discussed (12), this procedure specifically detects 8-OH-dG, which is the only modified nucleotide retained in chromatograms developed in acidic medium. To avoid artifacts that may result from radiation-induced oxidation of guanine, a selective hydrolysis of 7R,8S,9S-trihydroxy-10R-(N_2 -deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo(a)pyrene was achieved by treating depolymerized DNA with 90% trifluoroacetic acid before the ^{32}P postlabeling reaction (12). The results were expressed as 8-OH-dG/ 10^5 nucleotides and were generated in two separate experiments, each one assessing duplicate-pooled DNA samples.

Detection of Hb Adducts. Hb adducts of 4-ABP and BPDE were evaluated at the University of Louisville by testing packaged RBC. 4-ABP and 4'-F-ABP were purchased from Fluka Chemika-Biochemika (Ronkonkoma, NY) and Sigma-Aldrich Chemical Co. (Milwaukee, WI), respectively. Trimethylamine in hexane was prepared by adding 1 g of trimethylamine hydrochloride (Fluka) to 2 ml of water, neutralizing the solution with NaOH, and extracting with 10 ml of hexane. The internal standard, 4'-F-ABP, was recrystallized from dichloromethane-hexane and used to prepare a stock solution of 25 ng/ml in 0.1 M HCl which was stored at 4°C. Pentafluoropropionic anhydride was purchased from Fluka. The RBC were washed three times with 0.9% NaCl and lysed by the addition of 15 ml of ice cold deionized water and 2 ml of toluene with vigorous shaking. After 15 min, the lysate was removed by centrifugation at $10,000 \times g$ for 20 min to remove cellular debris. Hb concentrations were determined by measuring the absorbance at 415 nm (oxyHb; extinction coefficient, 125 mm^{-1}).

4-ABP adducts were measured by GC-MS (13). Before extraction the Hb samples were spiked by adding 400 pg of the internal standard 4'-F-ABP. The Hb solution was made 0.1 M in NaOH and incubated for 2 h at room temperature. The hydrolysate was extracted twice with 15 ml of methylene chloride, treated with 10 μl of trimethylamine in hexane, derivatized by the addition of 5 μl of pentafluoropropionic anhydride, and evaporated under nitrogen. The residue was dissolved in 20 μl of hexane, and a 3- μl aliquot was injected into the GC-MS by using a Hewlett-Packard 5890 Series II GC connected to a 5971A mass selective detector. BPDE-Hb adducts were evaluated by means of a GC-MS procedure (14). Briefly, globin samples (3–5 mg) were hydrolyzed for 1 h with 0.3 M HCl at 80°C, extracted three times with equal volumes of water-saturated isomyl alcohol, and evaporated. Dried samples were dissolved in hexane (25 μl) and derivatized with a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine before GC-MS. Quantitation of benzo(a)pyrene tetrol was calculated based on an external standard curve. The samples from each rat were run either in eight replicates (4-ABP-Hb) or seven replicates (BPDE-Hb).

Evaluation of Cytogenetic Parameters. Slides of BAL cells were stained with a Giemsa 10% solution for 10 min. A total of 2000 PAM per sample were scored for the presence of MN and PN (≥ 2 nuclei/cell) PAM. Slides of bone marrow were stained with May-Grünwald-Giemsa according to the method of Schmid (15). A total of 3000 polychromatic erythrocytes (PCE) per sample were scored for the presence of MN cells. For cytogenetic analyses, a total of 448,000 cells were examined microscopically.

Statistical Analyses. The effects of exposure to ETS and treatments with chemopreventive agents were evaluated by Student's *t* test for unpaired data. Correlations between DNA adduct levels in different cell types and between different biomarkers were evaluated by using Spearman's and simple regression tests (16).

RESULTS

Food Intake and Body Weight Gain. The daily food intakes per rat during the 31 days of the experiment were not significantly different among sham-exposed rats [19.5 ± 1.2 g/rat/day (SD)] and ETS-exposed rats, either in the absence of chemopreventive agents (19.4 ± 0.9) or in the presence of NAC (19.5 ± 0.7), PEITC (19.5 ± 1.0), or 5,6-BF (18.9 ± 1.2). Compared with both sham-exposed and ETS-exposed rats, the overall food intake was slightly but significantly lower in ETS-exposed rats treated with OPZ (18.5 ± 1.6 , $P < 0.01$), 1,2-D3T (18.0 ± 2.6 , $P < 0.01$), or

OPZ + NAC (17.5 ± 2.2 , $P < 0.001$). The dietary consumption in the ETS + OPZ + NAC group was significantly lower than either in the ETS + OPZ group ($P < 0.05$) or the ETS + NAC group ($P < 0.001$).

Table 1 reports the body weights of the eight rats belonging to each one of the eight experimental groups, measured at weekly intervals during the 4 weeks of exposure to ETS. A 23.4% body weight gain was recorded in the group of sham-exposed rats; whereas in ETS-exposed rats, in the absence of chemopreventive agents, the overall gain was 12.8%. The body weight in this group was significantly lower than in sham-exposed rats after 3 and 4 weeks of exposure to ETS. Similar body weight gains were observed in ETS-exposed rats receiving most chemopreventive agents, i.e., OPZ (11.7%), NAC (7.7%), NAC plus OPZ (9.3%), PEITC (7.3%), and 5,6-BF (10.0%). The group of rats treated with 1,2-D3T was the only one in which there was no body weight gain during the period of exposure to ETS, the body weight being significantly lower, at all times, than in sham-exposed rats and in ETS-exposed rats without chemopreventive agents.

Actual Intake of Chemopreventive Agents. The actual intake of each chemopreventive agent was calculated by taking into account the concentration of each agent, either in the diet (OPZ, 1,2-D3T, PEITC, and 5,6-BF) or in drinking water (NAC), the daily consumption of food or water (data not shown), and the body weights of rats (Table 1). As shown in Table 2, the actual intakes were, depending on the week, 21.4–26.1 mg/day/kg body weight for 1,2-D3T, 30.8–31.3 for PEITC, and 27.4–30.5 for 5,6-BF. The intake of OPZ was 20.5–25.2 mg/day/kg body weight when given alone and 19.3–24.8 when given in combination with NAC. The intake of NAC was 1.1–1.3 g/day/kg body weight when given alone, and 1.0–1.3 when given in combination with OPZ.

Modulation of ^{32}P -postlabeled DNA Adducts. The autoradiographic patterns were similar in BAL cells, tracheal epithelium, lung, and heart. In particular, up to four discrete spots could be distinguished in sham-exposed rats; two spots, close to the origin of the chromatogram, were identified as c1 and c2; two spots, characterized by a greater mobility, were identified as 1 and 3 (not shown). Spot 1 was detected in all cell types, whereas spot 3 was detectable only in the lung of sham-exposed rats. In ETS-exposed rats, 3 spots (1–3) were evident and consistently detected, and spots c1 and c2 were replaced by a massive DRZ (Fig. 1A).

Table 3 summarizes the results obtained in the eight experimental groups. In sham-exposed rats, the levels of ^{32}P -postlabeled DNA adducts ranked as follows: heart > lung > BAL cells > tracheal epithelium. Exposure to ETS resulted in a marked increase of spots 1, 2, and 3 and in the appearance of a DRZ. The ratio of total DNA adduct levels in ETS-exposed versus sham-exposed rats was 14.5 in the tracheal epithelium, 10.3 in BAL cells, 8.5 in lung, and 6.5 in heart.

With the exception of OPZ, all tested chemopreventive agents were successful in inhibiting the formation of ETS-related DNA adducts to a significant extent (Table 3). Table 4 shows at a glance the effects produced by chemopreventive agents on the monitored end points. The modulation of DNA adducts in BAL cells was least evident after the statistical analysis, because the necessity to work with pooled samples lowered the degrees of freedom. In any case, it is clear from both Table 3 and Table 4 that OPZ failed to affect DNA adduct formation in all types of cells. 1,2-D3T was ineffective in BAL cells but significantly decreased total DNA adducts and DRZ in the lung and tracheal epithelium; in addition, it inhibited spot 2 in the heart. PEITC, 5,6-BF, NAC, and its combination with OPZ displayed a potent and broad-spectrum inhibition of the ETS-induced nucleotide alterations.

Modulation of DNA adducts by chemopreventive agents was cal-

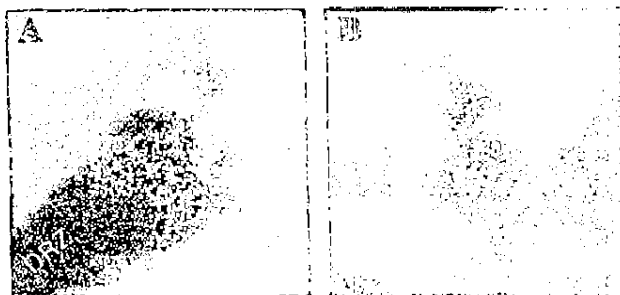


Fig. 1. Autoradiographic patterns of ^{32}P -postlabeled DNA adducts in the lung of ETS-exposed rats, as detected by using two chromatographic conditions, referred to as System A and System B (see text).

culated as $[100 - (C - A/B - A) \times 100]$, where A is DNA adduct levels in sham-exposed rats, B in ETS-exposed rats without chemopreventive agents, and C in ETS-exposed rats with each chemopreventive agent. The resulting rank of potency in inhibiting ETS-related total DNA adduct was as follows: BAL cells, OPZ + NAC (75.3%) > NAC (72.7%) > 5,6-BF (67.9%) > PEITC (57.6%) > 1,2-D3T (46.1%) > OPZ (14.7%, not significant); tracheal epithelium, OPZ + NAC (65.4%) > NAC (63.0%) > 5,6-BF (62.6%) > PEITC (59.1%) > 1,2-D3T (42.4%) > OPZ (37.1%, not significant); lung cells, OPZ + NAC (75.6%) > 5,6-BF (71.1%) > NAC (59.6%) > PEITC (52.9%) > 1,2-D3T (23.6%) > OPZ (-4.9%, i.e., a nonsignificant increase); heart, 5,6-BF (60.2%) > PEITC (46.3%) > OPZ + NAC (41.9%) > NAC (36.2%) > 1,2-D3T (25.2%) > OPZ (10.2%, not significant).

Thus, combination of NAC with OPZ was consistently more efficient than NAC alone, but only in the lung inhibition of total DNA adduct levels by OPZ + NAC was it greater than the sum of the effects produced individually by the OPZ/NAC combination. Indeed, DNA adduct levels in the lung of ETS-exposed rats receiving OPZ + NAC (8.5 ± 3.6 adducts/ 10^4 nucleotides; see Table 3) were not only much lower than in rats receiving OPZ alone (25.5 ± 5.2 , $P < 0.001$) but also appreciably lower than in rats receiving NAC alone (12.1 ± 3.4); this difference was statistically significant ($P = 0.05$).

The effects of exposure to ETS and treatment with chemopreventive agents on DNA adduct levels were quite similar in the different types of investigated cells. In fact, DNA adduct levels in the lung, tracheal epithelium, BAL cells, and heart among the eight experimental groups were highly correlated, as follows: lung *versus* tracheal epithelium: total adducts, $r = 0.955$, $P < 0.001$; DRZ, $r = 0.977$, $P < 0.001$; spot 1, $r = 0.358$, not significant; spot 2, $r = 0.877$, $P < 0.01$; spot 3, $r = 0.860$, $P < 0.01$; *versus* BAL cells: total adducts, $r = 0.893$, $P < 0.01$; DRZ, $r = 0.990$, $P < 0.001$; spot 1, $r = 0.583$, not significant; spot 2, $r = 0.818$, $P < 0.01$; spot 3, $r = 0.568$, not significant; lung *versus* heart: total adducts, $r = 0.828$, $P < 0.01$; DRZ, $r = 0.855$, $P < 0.01$; spot 1, $r = 0.795$, $P < 0.05$; spot 2, $r = 0.710$, $P < 0.05$; spot 3, $r = 0.843$, $P < 0.01$; tracheal epithelium *versus* BAL cells: total adducts, $r = 0.988$, $P < 0.001$; DRZ, $r = 0.985$, $P < 0.001$; spot 1, $r = 0.660$, not significant; spot 2, $r = 0.858$, $P < 0.01$; spot 3, $r = 0.639$, not significant; tracheal epithelium *versus* heart: total adducts, $r = 0.945$, $P < 0.001$; DRZ, $r = 0.922$, $P < 0.001$; spot 1, $r = 0.539$, not significant; spot 2, $r = 0.856$, $P < 0.01$; spot 3, $r = 0.918$, $P < 0.001$; BAL cells *versus* heart: total adducts, $r = 0.893$, $P < 0.01$; DRZ, $r = 0.864$, $P < 0.01$; spot 1, $r = 0.527$, not significant; spot 2, $r = 0.824$, $P < 0.05$; spot 3, $r = 0.589$, not significant.

Comparison of Two Chromatographic Conditions for Evaluating Inhibition of DNA Adducts in the Lung by Oltipraz and/or N-Acetyl-L-cysteine. The observed effects of NAC and OPZ on smoke-induced formation of DNA adducts, as described in the previous section, were opposite to those found in another laboratory (see "Discussion"). This prompted us to perform additional experiments aimed at assessing whether these discrepant findings may be ascribed to the different conditions followed for the TLC separation of ^{32}P -postlabeled DNA adducts. When using System A, which was the one used in all other experiments of the present study, the data (not shown) were fully comparable with those reported in Table 3.

DNA adduct patterns in the lung, as detected by using System B, were different from those detected by using System A, in that no DRZ was visible, and four discrete spots were detected, the chromatographic positions of which did not correspond to the three spots detected with System A (Fig. 1). Total DNA adduct levels in the lung of ETS-exposed rats were 15 times lower than those detected with System A, and inhibition patterns were also completely different. In fact, when using System B, NAC treatment did not affect DNA adduct levels and patterns, whereas OPZ produced an attenuation of DNA adducts (data not shown). A further decrease in DNA adduct levels was produced by combined treatment with OPZ and NAC, which significantly decreased both total DNA adducts and spot 1. Furthermore, the combined NAC/OPZ treatment significantly decreased total DNA adducts ($P < 0.05$) as compared with treatment with OPZ alone as well as total DNA adducts ($P < 0.01$), spot 1 ($P < 0.05$), and spot 2 ($P < 0.01$) as compared with treatment with NAC alone (data not shown). NAC did not inhibit at all total DNA adducts, OPZ produced a 35.3% inhibition, and OPZ + NAC produced a 58.8% inhibition. Therefore, although individually the two drugs yielded opposite effects under the two chromatographic conditions, their combined treatment consistently yielded more than additive effects.

Modulation of Oxidative DNA Damage. Oxidative DNA damage was evaluated in lung cells by measuring 8-OH-dG levels. The results reported in Table 3 are means \pm SD of the values recorded in two separate experiments, each one assaying in duplicate lung DNA pooled within each group of rats. Exposure of rats to ETS resulted in a 2.2-fold increase of 8-OH-dG as compared with sham-exposed rats. As also shown in Table 4, all tested chemopreventive agents were successful in preventing the ETS-induced oxidative damage of pulmonary DNA, to such an extent that 8-OH-dG levels were comparable with those observed in sham-exposed rats.

The profiles of 8-OH-dG levels in lung DNA in the eight treatment groups were poorly correlated with DNA adduct levels in the same cells. The only significant correlation was between 8-OH-dG and DRZ ($r = 0.720$, $P < 0.05$), which indicates a parallel effect of exposure to ETS and treatment with chemopreventive agents on this marker of oxidative DNA damage and the nucleotide alterations contributing to formation of DRZ.

Modulation of Hb Adducts. The results of Hb adduct analyses are summarized in Table 3, where the data are reported as means \pm SD within the eight rats composing each experimental group. The samples from each rat were run either in seven (BPDE-Hb) or eight (4-ABP-Hb) replicates. Exposure of rats to ETS resulted in powerful increases of both 4-ABP-Hb adducts (46.9-fold) and BPDE-Hb adducts (29.6-fold). All chemopreventive agents produced significant decreases of ETS-induced Hb adducts (Tables 3 and 4). In general, inhibition of 4-ABP-Hb adducts was more pronounced than inhibition of BPDE-Hb adducts. In particular, the rank of potency in inhibiting ETS-related 4-ABP-Hb adducts, calculated as indicated for DNA adducts, was: OPZ + NAC (68.0%) > 5,6-BF (52.3%) > PEITC (50.8%) > NAC (42.7%) > 1,2-D3T (33.8%) > OPZ (33.4%). The rank for BPDE-Hb adducts was:

Table 3. Modulation of molecular and cytogenetic endpoints by chemopreventive agents in rat organs, tissues, and cells

Endpoint	Treatment groups							
	Sham	ETS	ETS + OPZ	ETS + 1,2-D3T	ETS + NAC	ETS + NAC + OPZ	ETS + PEITC	ETS + 5,6-BF
DNA adducts in BAL cells^a								
Spot 1	0.7 ± 0.2 ^b	2.1 ± 0.7 ^c	2.0 ± 0.5 ^c	1.1 ± 0.3 ^c	0.9 ± 0.2 ^{c,e}	0.8 ± 0.1 ^{c,e}	0.5 ± 0.1 ^c	2.0 ± 0.4 ^{c,f}
Spot 2	0.0 ± 0.0	0.6 ± 0.2 ^c	0.4 ± 0.1 ^c	0.2 ± 0.1 ^c	0.2 ± 0.0 ^{c,e}	0.1 ± 0.1 ^{c,h}	0.1 ± 0.1 ^{c,h}	0.2 ± 0.0 ^{c,h}
Spot 3	0.0 ± 0.0	0.5 ± 0.1 ^c	0.3 ± 0.1 ^c	0.3 ± 0.1 ^c	0.2 ± 0.1 ^{c,h}	0.1 ± 0.0 ^{c,h}	0.1 ± 0.1 ^{c,h}	0.4 ± 0.2 ^{d,h}
DRZ/spots c1 and c2	2.2 ± 0.6	26.8 ± 3.0 ^f	23.3 ± 3.0 ^f	19.9 ± 3.0 ^f	9.0 ± 1.8 ^{g,h}	8.6 ± 2.5 ^{d,h}	13.7 ± 3.3 ^{c,h}	9.0 ± 2.1 ^{c,h}
Total	2.9 ± 0.7	30.0 ± 3.2 ^f	26.0 ± 3.1 ^f	17.5 ± 3.3 ^f	10.3 ± 2.2 ^{c,h}	9.6 ± 3.2 ^{c,h}	14.4 ± 3.2 ^{c,h}	11.6 ± 2.4 ^{d,h}
DNA adducts in the tracheal epithelium^a								
Spot 1	1.2 ± 0.7	2.0 ± 1.1	1.6 ± 0.8	1.6 ± 0.7	1.5 ± 0.7	1.4 ± 0.6	1.8 ± 0.6	2.3 ± 0.6
Spot 2	0.0 ± 0.0	0.8 ± 0.5 ^c	0.7 ± 0.2 ^c	0.5 ± 0.4 ^c	0.5 ± 0.3 ^d	0.4 ± 0.4 ^d	0.5 ± 0.3 ^c	0.3 ± 0.2 ^{d,e}
Spot 3	0.0 ± 0.0	0.7 ± 0.7 ^c	0.6 ± 0.3 ^c	0.4 ± 0.4 ^c	0.3 ± 0.2 ^{d,h}	0.3 ± 0.3 ^{d,f}	0.4 ± 0.3 ^{d,e}	0.2 ± 0.2 ^{d,h}
DRZ/spots c1 and c2	0.7 ± 0.4	24.1 ± 5.0 ^f	20.3 ± 3.1 ^f	16.4 ± 4.8 ^{g,h}	9.1 ± 2.7 ^{g,h}	8.8 ± 2.7 ^{g,h}	9.7 ± 1.7 ^{c,h}	8.7 ± 2.5 ^{c,h}
Total	1.9 ± 1.0	27.6 ± 5.1 ^f	23.2 ± 3.4 ^f	16.7 ± 4.6 ^{g,h}	11.4 ± 3.1 ^{c,h}	10.8 ± 2.2 ^{c,h}	12.4 ± 2.1 ^{c,h}	11.3 ± 2.2 ^{c,h}
DNA adducts in the lung^a								
Spot 1	1.7 ± 0.7	6.3 ± 1.8 ^f	7.5 ± 2.6 ^f	5.8 ± 2.3 ^f	4.0 ± 1.7 ^{d,e}	3.0 ± 1.5 ^{g,h}	4.7 ± 1.7 ^c	3.5 ± 2.5 ^{c,f}
Spot 2	0.0 ± 0.0	2.2 ± 0.6 ^f	2.6 ± 0.6 ^f	2.0 ± 0.8 ^f	1.3 ± 0.6 ^{c,e}	0.8 ± 0.5 ^{c,h}	1.0 ± 0.7 ^{c,h}	0.6 ± 0.2 ^{c,h}
Spot 3	0.2 ± 0.2	2.9 ± 0.8 ^f	3.2 ± 0.9 ^f	3.0 ± 1.1 ^f	1.6 ± 0.6 ^{c,h}	1.2 ± 0.4 ^{c,h}	1.3 ± 0.7 ^{c,h}	1.0 ± 0.7 ^{d,h}
DRZ/spots c1 and c2	1.2 ± 0.6	14.2 ± 4.2 ^f	13.3 ± 3.8 ^f	9.4 ± 2.2 ^{c,f}	5.0 ± 1.2 ^{c,h}	3.5 ± 1.6 ^{d,h}	6.6 ± 2.3 ^{c,h}	4.4 ± 2.0 ^{c,h}
Total	3.0 ± 1.1	25.5 ± 5.2 ^f	26.6 ± 4.1 ^f	20.2 ± 4.5 ^{c,f}	12.1 ± 3.4 ^{c,h}	8.5 ± 3.3 ^{c,h}	13.6 ± 3.6 ^{c,h}	9.5 ± 5.0 ^{c,h}
DNA adducts in the heart^a								
Spot 1	2.3 ± 1.3	4.5 ± 1.2 ^f	5.4 ± 2.4 ^f	4.8 ± 1.8 ^d	4.1 ± 1.3 ^f	4.3 ± 1.0 ^f	4.5 ± 1.4 ^f	4.7 ± 1.7 ^d
Spot 2	0.0 ± 0.1	1.7 ± 1.1 ^f	0.8 ± 0.8 ^f	0.6 ± 0.4 ^{d,e}	0.3 ± 0.3 ^g	0.3 ± 0.3 ^{g,h}	0.4 ± 0.2 ^{c,g}	0.3 ± 0.2 ^g
Spot 3	0.0 ± 0.0	1.4 ± 1.0 ^f	0.9 ± 0.6 ^f	0.7 ± 0.3 ^f	0.4 ± 0.5 ^f	0.5 ± 0.4 ^{c,f}	0.6 ± 0.4 ^{c,f}	0.2 ± 0.3 ^{g,h}
DRZ/spots c1 and c2	2.1 ± 1.2	21.6 ± 4.9 ^f	19.6 ± 2.5 ^f	16.7 ± 1.9 ^{c,f}	15.4 ± 3.9 ^{c,f}	13.5 ± 1.2 ^{c,h}	12.2 ± 2.4 ^{c,h}	14.1 ± 3.2 ^{c,h}
Total	4.5 ± 1.2	29.1 ± 5.4 ^f	26.8 ± 3.9 ^f	22.9 ± 3.1 ^{c,f}	20.2 ± 4.5 ^{c,h}	18.8 ± 2.3 ^{c,h}	17.7 ± 2.9 ^{c,h}	19.3 ± 4.4 ^{c,h}
Oxidative damage to lung DNA								
8-OH-dG ^a	1.7 ± 0.7	3.7 ± 0.7 ^d	1.9 ± 0.6 ^f	1.7 ± 0.7 ^c	1.5 ± 0.4 ^f	1.3 ± 0.2 ^f	1.5 ± 0.9 ^c	1.3 ± 0.7 ^c
Hemoglobin adducts								
4-ABP ^a	2.5 ± 0.1	116.3 ± 2.0 ^f	78.2 ± 0.7 ^{c,h}	77.8 ± 1.0 ^{c,h}	67.7 ± 0.9 ^{c,h}	38.9 ± 0.1 ^{c,h}	58.5 ± 1.2 ^{c,h}	56.8 ± 3.2 ^{c,h}
BPDE ^a	2.2 ± 0.1	65.1 ± 2.8 ^f	49.9 ± 1.1 ^{c,h}	47.7 ± 1.0 ^{c,h}	47.1 ± 1.3 ^{c,h}	30.6 ± 0.2 ^{c,h}	49.5 ± 0.8 ^{c,h}	49.5 ± 1.3 ^{c,h}
Cytogenetic damage								
MN PAM (%)	0.8 ± 0.5	2.5 ± 0.7 ^c	2.1 ± 0.6 ^c	5.2 ± 1.3 ^{c,h}	1.8 ± 0.7 ^{d,e}	1.6 ± 0.7 ^{c,f}	0.9 ± 0.4 ^h	1.6 ± 0.8 ^{c,f}
PN PAM (%)	63.1 ± 14.9	107.3 ± 28.6 ^d	97.5 ± 78.9 ^f	83.6 ± 19.6	70.1 ± 13.5 ^d	79.9 ± 16.2 ^{c,f}	66.8 ± 18.7 ^e	72.9 ± 27.3 ^e
MN PCE (%)	0.8 ± 0.5	1.4 ± 0.6 ^f	1.3 ± 0.6	1.3 ± 0.6 ^f	0.8 ± 0.5 ^c	0.8 ± 0.5 ^c	0.6 ± 0.4 ^h	0.5 ± 0.3 ^h

^a Adducts/10⁶ nucleotides. Chromatographic System A was used (see text).^b All results are means ± SD (see text).^c $P \leq 0.05$, ^d $P \leq 0.01$, and ^e $P \leq 0.001$, compared with sham-exposed rats.^f $P \leq 0.05$, ^g $P \leq 0.01$, and ^h $P \leq 0.001$, compared with ETS-exposed rats, in the absence of chemopreventive agents.^a 8-OH-dG/10⁶ nucleotides.^f pmol 4-ABP/mg globin or pmol BPDE/mg globin.

OPZ + NAC (54.8%) > NAC (28.6%) > 1,2-D3T (27.7%) > 5,6-BF and PEITC (24.8%) > OPZ (24.2%).

Thus, combination of NAC with OPZ was by far the most effective treatment, yielding a 68.0% inhibition of 4-ABP-Hb adducts versus 76.1% resulting from the sum of the individual effects, and an observed 54.8% inhibition of BPDE-Hb adducts versus an expected 52.8%. Inhibition of both 4-ABP-Hb and BPDE-Hb adducts by the NAC/OPZ combination was significantly greater than inhibition produced by either chemopreventive agent individually ($P < 0.001$).

There was a high and significant correlation ($r = 0.937$, $P < 0.001$) between levels of 4-ABP-Hb and BPDE-Hb levels among the eight experimental groups. Moreover, the levels of both 4-ABP-Hb and BPDE-Hb adducts among the eight groups were highly correlated with the levels of DNA adducts (total, DRZ, spot 1, spot 2, and spot 3) in BAL cells, tracheal epithelium, lung, and heart (data not shown).

Modulation of Cytogenetic Parameters. Exposure of rats to ETS resulted in a significant increase of MN PAM (3.1-fold) and PN PAM (1.7-fold), and of MN PCE (1.8-fold) in bone marrow. Chemopreventive agents had variable effects on these cytogenetic parameters (Tables 3 and 4). The dithiolthiones OPZ and 1,2-D3T failed to inhibit ETS-related alterations, and 1,2-D3T even enhanced to a significant extent the frequency of MN PAM, which was 2.1-fold higher than in ETS-exposed rats in the absence of any chemopreventive agent. In contrast, PEITC, 5,6-BF, NAC, and its combination with OPZ significantly inhibited all cytogenetic alterations induced by ETS.

The rank of potency in inhibiting ETS-related cytogenetic alterations was as follows: MN PAM, PEITC (89.1%) > OPZ + NAC (53.7%) > 5,6-BF (49.7%) > NAC (42.9%); PN PAM, PEITC (91.5%) > NAC (84.2%) > 5,6-BF (77.8%) > OPZ + NAC (62.0%);

MN PCE, PEITC and 5,6-BF (both 100%) > OPZ + NAC (93.5%) > NAC (87.1%).

DISCUSSION

Whole-body exposure of Sprague Dawley rats to ETS consistently resulted in significant alterations of all of the investigated parameters, which covered a range of early biological modifications, from molecular damage to cytogenetic alterations. Promutagenic lesions were strongly enhanced in smoke-exposed rats, as shown by 6.5- to 15-fold increases of total DNA adduct levels in lung, heart, tracheal epithelium, and BAL cells. Moreover, the levels of two specific Hb adducts related to smoke exposure (BPDE and 4-ABP) were increased 30-47-fold. The intensity of oxidative DNA damage (8-OH-dG levels) in lung cells as well as cytogenetic alterations both in the lower respiratory tract (PAM) and in bone marrow (PCE) were also significantly increased after exposure of rats to ETS, but to a less dramatic extent. These results are in agreement with the conclusions of our previous studies concerning the formation and stability of ETS-related DNA adducts in various rat tissues (7), induction of 8-OH-dG in lung cells (7), formation of 4-ABP- and BPDE-Hb adducts,⁵ and induction of cytogenetic damage in smoke-exposed rodents (reviewed in Ref. 17). In the same papers, we thoroughly discussed the possible pathogenetic meaning of these biological alterations. Taken together, these results converge in providing further evidence that cigarette smoke is a powerful inducer of genotoxic damage in multiple organs of exposed rodents, which contrasts with difficulties encountered in reproducing smoke-related lung carcinogenicity in animal models (3, 18).

The five chemopreventive agents, administered p.o., and the com-

Table 4. Summary of the effects produced by chemopreventive agents on ETS-induced biomarkers

Endpoint	Treatment groups					
	ETS + OPZ	ETS + DTT	ETS + NAC	ETS + NAC + OPZ	ETS + PEITC	ETS + BF
DNA adducts in BAL cells						
Spot 1	— ^a	—	↓	↓	—	↓
Spot 2	—	—	↓	↓	↓	↓
Spot 3	—	—	↓	↓	↓	↓
DRZ	—	↓	↓	↓	↓	↓
Total	—	↓	↓	↓	↓	↓
DNA adducts in the tracheal epithelium						
Spot 1	—	—	—	—	—	—
Spot 2	—	—	↓	↓	↓	↓
Spot 3	—	—	↓	↓	↓	↓
DRZ	—	↓	↓	↓	↓	↓
Total	—	↓	↓	↓	↓	↓
DNA adducts in the lung						
Spot 1	—	—	↓	↓	—	↓
Spot 2	—	—	↓	↓	↓	↓
Spot 3	—	—	↓	↓	↓	↓
DRZ	—	↓	↓	↓	↓	↓
Total	—	↓	↓	↓	↓	↓
DNA adducts in the heart						
Spot 1	—	—	—	—	—	—
Spot 2	—	↓	↓	↓	↓	↓
Spot 3	—	↓	↓	↓	↓	↓
DRZ	—	↓	↓	↓	↓	↓
Total	—	↓	↓	↓	↓	↓
Oxidative DNA damage in lung						
8-OH-dG	↓	↓	↓	↓	↓	↓
Hemoglobin adducts						
4-ABP	↓	↓	↓	↓	↓	↓
BPDE	↓	↓	↓	↓	↓	↓
Cytogenetic effects						
MN PAM	—	↑	↓	↓	↓	↓
PN PAM	—	—	↓	↓	↓	↓
MN PCE	—	—	↓	↓	↓	↓

^a ↑, significant increase compared with rats exposed to ETS only; ↓, significant decrease compared with rats exposed to ETS only; —, no significant variation.

bination of two of them were evaluated only in conjunction with exposure to ETS and at one dose for each agent. Agents and doses were selected based on our previous studies, data in the literature, and past or ongoing National Cancer Institute-sponsored studies (see, e.g., Refs. 19–32). OPZ and 1,2-D3T were administered at doses close to their maximum tolerated doses. NAC was used at a relatively high albeit nontoxic dose. PEITC was used at a dose that was highly effective in prior studies with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (19).

Under our experimental conditions, the two dithiolethiones administered to ETS-exposed rats displayed some adverse effects. In particular, 1,2-D3T was frankly toxic, as shown not only by the impaired food intake but especially by the striking lack of body weight gain. In addition, this treatment resulted in a marked and significant increase of MN in PAM cells populating terminal airways. The only genotoxic effect available to our files, concerning 1,2-D3T or its substituted analogue OPZ, is an increase of sister chromatid exchanges induced by OPZ in mammalian cultured cells at relatively low concentrations (0.1 µg/ml medium) (20). Certainly, the selective genotoxic effect produced by 1,2-D3T in PAM and not in bone marrow PCE of ETS-exposed rats and the systemic toxicity of this treatment in terms of lack of body weight gain warrant further studies, especially aimed at assessing whether these adverse effects of 1,2-D3T require the concomitant exposure to smoke or reflect a genotoxic potential of 1,2-D3T *per se* in PAM.

It is well documented that dithiolethiones possess antigenotoxic and anticarcinogenic properties in a variety of experimental test systems (6, 21, 22). Among other mechanisms, these compounds act as monofunctional inducers of phase II enzymes (21). In the present study, OPZ alone failed to significantly affect the ETS-related formation of DNA adducts and induction of cytogenetic damage, whereas it significantly attenuated the pulmonary oxidative DNA damage and

4-ABP-Hb and BPDE-Hb adducts. Apart from its systemic toxicity and genotoxicity to PAM, 1,2-D3T significantly decreased ETS-induced total DNA adducts and DRZ in BAL cells, tracheal epithelium, and lung and total adducts, DRZ and spot 2 in the heart, oxidative damage to lung DNA, and Hb adducts to 4-ABP and BPDE.

The three remaining chemopreventive agents, i.e., 5,6-BF, PEITC, and NAC, were the most potent inhibitors of all of the investigated alterations resulting from exposure of rat to ETS. 5,6-BF is a broad-spectrum inducer of Phase I and Phase II activities involved in the metabolism of xenobiotics (23). We previously showed that this agent is profound inhibitor of 7,12-dimethylbenz(a)anthracene-induced DNA adducts and mammary carcinogenesis in rats (24). PEITC, like other organic isothiocyanates, inhibits Phase I enzymes involved in carcinogen activation and at the same time induces Phase II enzymes that accelerate cellular disposal of activated carcinogens (25). This effect has been also reported to occur with typical components of cigarette smoke, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (26). It has been postulated that enzyme induction is signaled by free isothiocyanates, the intracellular concentrations of which are inversely related to cellular GSH stores because isothiocyanates are readily and reversibly converted to dithiocarbamates (27). Either *per se* or as a precursor of intracellular cysteine and GSH, NAC is a weak inducer of both Phase I and Phase II enzymes and exerts antigenotoxic and anticarcinogenic effects through a variety of mechanisms, among which are nucleophilicity and scavenging of reactive oxygen species (28, 29). NAC has previously been found to significantly attenuate cytogenetic alterations in Sprague Dawley rats (30) and C57BL × DBA/2 F₁ mice (17) as well as adducts to either mitochondrial DNA (31) or nuclear DNA (10, 11, 28, 32) in various tissues of Sprague Dawley rats exposed whole body to mainstream cigarette smoke.

In general, there was a close parallelism and a significant correlation in the modulation of total DNA adducts, DRZ, and the three individual spots among the four types of cell populations. Formation of DNA adducts in the mixed cell lung population, in tracheal epithelium, and in BAL cells reflects an increased risk of developing lung cancer and perhaps certain chronic obstructive pulmonary diseases, whereas formation of DNA adducts in perinatal cardiac myocytes has tentatively been associated with smoke-related cardiomyopathies (33). However, both PEITC and NAC (34) and a mixture of PEITC and benzyl isothiocyanate (35) failed to inhibit the weak tumorigenic response induced in the lung of A/J mice exposed to ETS for 5 months, followed by 4 months of recovery in filtered air. A good parallelism and a significant correlation also occurred in the modulation of 4-ABP-Hb and BPDE-Hb adducts, which in turn were both highly correlated with total DNA adducts, DRZ, and the three individual spots in all investigated cell types. GSH and Hb compete for reaction with nitrosobiphenyl, a reactive metabolite of 4-ABP that covalently binds Hb (36), which explains the observed protective effect of precursors of intracellular GSH, such as NAC, which even in nonsmoking humans has been shown to lower 4-ABP-Hb adduct levels (37). These patterns should be taken into account in the design and choice of biomarkers in Phase II chemopreventive trials in smokers.

The efficiency of NAC in inhibiting DNA adducts, contrasting with the lack of protection by OPZ, deserves particular comments. In fact, opposite results have been obtained in another laboratory, in which NAC failed to inhibit the formation of lipophilic DNA adducts in tissues of Sprague Dawley rats exposed whole body to sidestream smoke (38), whereas OPZ was effective in the same model (39). The results of comparative analyses provided evidence that the above discrepancies are methodological and namely depend on the chromatographic conditions used in D3 for the separation of ^{32}P -postlabeled DNA adducts. The problem is that, as evaluated in the comparative analyses reported in a previous study (7) and confirmed herein, System B yields a much lower amount of DNA adducts than System A and fails to detect the massive DRZ which is the expression of the multitude of DNA-binding agents present in cigarette smoke. Because System B underestimates the overall genotoxic potential of cigarette smoke, evaluation of the efficacy of chemopreventive agents by using this procedure is made only toward a few selected components of this complex mixture. In our experimental model, NAC was much more effective than OPZ in modulating all ETS-related biomarkers. In humans, NAC administration was found to decrease DNA adduct levels in BAL cells of Dutch smokers (40), and decreased the load of smoke-related urinary mutagens in the majority of the Italian subjects thus far examined (41). In contrast, OPZ failed to modulate the excretion of urinary mutagens in Chinese smokers.⁶ Therefore, on the whole, the data available in both humans and experimental animals suggest that NAC compares favorably with OPZ in attenuating smoke-related biomarkers. Nevertheless, the findings that administration of OPZ to rats inhibits smoke-induced lipophilic DNA adducts in the lung, as shown by Arif *et al.* (39) and confirmed in this study, and that OPZ can block *in vitro* alterations of human fetal lung DNA caused by cigarette smoke condensate (42) deserve further attention on some possible protective role of OPZ in smoke carcinogenesis.

An interesting issue was the outcome of the combination of NAC (given with drinking water) and OPZ (given with the diet) in modulating the monitored biomarkers, due to the distinctive and possibly

complementary mechanisms of action of these drugs (22, 28, 29). A positive interaction between NAC and OPZ was observed for DNA adducts in the lung and both 4-ABP- and BPDE-Hb adducts.

In conclusion, the results of the present study indicate that chemopreventive agents working with different mechanisms of action can prevent a variety of biomarkers involved in early stages of the smoke-related pathogenesis of lung cancer and possibly of other chronic degenerative diseases in which alterations of these biomarkers can be detected (33, 43). The positive interactions observed by associating an aminothiol and a dithiothione are encouraging in the framework of the expanding field of combined chemoprevention.

REFERENCES

- Doll, R., and Peto, R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.*, 66: 1192-1308, 1981.
- United States Department of Health and Human Services. Reducing the health consequences of smoking, 25 years of progress. In: Office on Smoking and Health DHSS Publication No. CDC-89-8411. pp. 43-54. Washington, DC: Government Printing Office, 1989.
- IARC. Tobacco smoking. IARC Monographs on the Evaluation of the Carcinogenic Risk of Humans. Lyon, France: IARC, 1986.
- Buiatti, E., Gaddes, M., and Amiani, S. Epidemiology of lung cancer. *Ann. Ist. Sper. Sanità*, 32: 133-144, 1996.
- Marshall, E. Cancer warriors claim a victory. *Science* (Washington DC), 279: 1842-1843, 1998.
- Steele, V. E., Moon, R. C., Lahet, R. A., Grubbs, C. J., Reddy, B. S., Wargovich, M., McCormick, D. L., Pereira, M. A., Crowell, I. A., and Bagheri, D. Preclinical efficacy evaluation of potential chemopreventive agents in animal carcinogenesis models: methods and results from the NCI Chemoprevention Drug Development Program. *J. Cell Biochem.*, 20 (Suppl.): 32-54, 1994.
- Izzotti, A., Bagnasco, M., D'Agostini, F., Cartiglia, C., Lubet, R. A., Kelloff, G. J., and De Flora, S. Formation and persistence of nucleotide alterations in rats exposed whole-body to environmental cigarette smoke. *Carcinogenesis* (Lond.), 20: 1499-1505, 1999.
- Teague, S. V., Pinkerton, K. E., Goldsmith, M., Gebremichael, A., Chang, S., Jenkins, R. A., and Moneyham, J. H. Sidestream cigarette smoke generation and exposure system for environmental tobacco smoke studies. *Inhal. Toxicol.*, 6: 79-93, 1994.
- Gupta, R. C. Nonrandom binding of the carcinogen *N*-hydroxy-2-acetylaminofluorene to repetitive sequences of rat liver DNA *in vivo*. *Proc. Natl. Acad. Sci. USA*, 81: 6943-6947, 1984.
- Izzotti, A., Balansky, R. M., Coscia, N., Scatolini, L., D'Agostini, F., and De Flora, S. Chemoprevention of smoke-related DNA adduct formation in rat lung and heart. *Carcinogenesis* (Lond.), 13: 2187-2190, 1992.
- Izzotti, A., D'Agostini, F., Bagnasco, M., Scatolini, L., Rovida, A., Balansky, R. M., Cesarone, C. F., and De Flora, S. Chemoprevention of carcinogen-DNA adducts and chronic degenerative diseases. *Cancer Res.*, 54: 1994s-1998s, 1994.
- Izzotti, A., Cartiglia, C., Tanager, M., De Flora, S., and Balansky, R. M. Age-related increases of 8-hydroxy-2'-deoxyguanosine and DNA protein crosslinks in mouse organs. *Mutat. Res.*, 446: 215-223, 1999.
- Pinarik-Godly, M. T., and Myers, S. R. HPLC and GC/MS determination of 4-aminobiphenyl hemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen *in utero*. *Toxicology*, 107: 209-217, 1995.
- Myers, S. R., Spinnato, J. A., and Pinarik, M. T. Chromatographic characterization of hemoglobin benz(a)pyrene-7,8-diol-9,10-epoxide adducts. *Fundam. Appl. Toxicol.*, 29: 94-101, 1996.
- Schmid, W. The micronucleus test. *Mutat. Res.*, 31: 9-15, 1975.
- Sokal, R., and Rohlf, F. J. Non parametric tests for association. In: R. Sokal and F. J. Rohlf (eds.), *Biometry*, pp. 593-601. New York: W. H. Freeman & Co., 1995.
- Balansky, R. M., D'Agostini, F., and De Flora, S. Induction, persistence and modulation of cytogenetic alterations in cells of smoke-exposed mice. *Carcinogenesis* (Lond.), 20: 1491-1497, 1999.
- Witschi, H., Esperito, I., Peake, J. L., Wu, K., Maronpot, R. R., and Pinkerton, K. E. The carcinogenicity of environmental tobacco smoke. *Carcinogenesis* (Lond.), 18: 575-586, 1997.
- Chung, F.-L., Kelloff, G., Steele, V., Pittman, B., Zang, E., Jiao, D., Rigotty, J., Choi, C.-I., and Rivenson, A. Chemopreventive efficacy of arylalkyl isothiocyanates and *N*-acetylcysteine for lung tumorigenesis in Fischer rats. *Cancer Res.*, 56: 772-778, 1996.
- Shubert, E. K., Jacobson, K. D., and Williams, J. R. Comparison of the Ames assay and the induction of sister chromatid exchanges: results with ten pharmaceuticals and five selected agents. *Cell Biol. Toxicol.*, 2: 379-390, 1986.
- Kensler, T. W., Grossman, J. D., and Roebuck, B. D. Chemoprotection by OPZ and other dithiothiones. In: L. W. Wattenberg, M. Lipkin, C. W. Boone, and G. J. Kelloff (eds.), *Cancer Chemoprevention*, pp. 203-226. Boca Raton, FL: CRC Press, 1992.
- Breinholt, V., and Dragsted, L. O. Antitumorigenic dithiothiones. *TemoNord*, Vol. 613, pp. 1-73. Copenhagen: EKSpressen, 1997.
- Boobis, A. R., Nebert, D. W., and Felton, J. S. Comparison of β -naphthoflavone and 3-methylcholanthrene as inducers of hepatic cytochrome(s) P448 and arylhydrocarbon-hydroxylase activity. *Mol. Pharmacol.*, 13: 259-268, 1978.

⁶ A. Camouzo, M. Bagnasco, C. Benicelli, C. Cartiglia, J.-B. Wang, B.-C. Zhang, Y.-R. Zhu, G.-S. Qian, P. A. Egner, L. P. Jacobson, T. W. Kensler, and S. De Flora. Oligonucleotide chemoprevention trial in Qidong, People's Republic of China: Results of urine genotoxicity assays as related to smoking habits, submitted for publication.

24. Izzotti, A., Camoirano, A., Cartiglia, C., Grubbs, C. J., Lubet, R. A., Kelloff, G. J., and De Flora, S. Patterns of DNA adduct formation in liver and mammary epithelial cells of rats treated with 7,12-dimethylbenz(a)anthracene, and selective effects of chemopreventive agents. *Cancer Res.*, 59: 4285-4290, 1999.
25. Prestera, T., Holtzclaw, W. D., Zhang, Y., and Talalay, P. Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. USA*, 90: 2965-2969, 1993.
26. Hecht, S. S. Chemoprevention of lung cancer by isothiocyanates. *Adv. Exp. Med. Biol.*, 401: 1-11, 1996.
27. Zhang, Y., and Talalay, P. Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer Res.*, 58: 4632-4639, 1998.
28. De Flora, S., Balansky, R. M., Bennicelli, C., Camoirano, A., D'Agostini, F., Izzotti, A., and Cesarone, C. F. Mechanisms of anticarcinogenesis: the example of *N*-acetylcysteine. In: C. Ioannides and D. F. V. Lewis (eds.), *Drugs, Diet and Diseases*, Vol. 1. Mechanistic Approach to Cancer, pp. 151-203. Hemel Hempstead, United Kingdom: Harwood Ellis, 1995.
29. De Flora, S., Cesarone, C. F., Balansky, R. M., Albini, A., D'Agostini, F., Bennicelli, C., Bagnasco, M., Camoirano, A., Scatolini, L., Rovida, A., and Izzotti, A. Chemopreventive properties and mechanisms of *N*-acetylcysteine: the experimental background. *J. Cell Biochem.*, 58 (Suppl. 22): 33-41, 1995.
30. Balansky, R., D'Agostini, F., and De Flora, S. Protection by *N*-acetylcysteine of the histopathological and cytogenetical damage produced by exposure of rats to cigarette smoke. *Cancer Lett.*, 64: 123-131, 1992.
31. Balansky, R., Izzotti, A., Scatolini, L., D'Agostini, F., and De Flora, S. Induction by carcinogens and chemoprevention by *N*-acetylcysteine of adducts to mitochondrial DNA in rat organs. *Cancer Res.*, 56: 1642-1647, 1996.
32. Izzotti, A., Balansky, R. M., Scatolini, L., Rovida, A., and De Flora, S. Inhibition by *N*-acetylcysteine of carcinogen-DNA adducts in the tracheal epithelium of rats exposed to cigarette smoke. *Carcinogenesis (Lond.)*, 16: 669-672, 1995.
33. De Flora, S., Izzotti, A., Randerath, K., Randerath, E., Bartsch, H., Nair, J., Balansky, R. M., van Schooten, F. J., Degan, P., Fronzi, G., Walsh, D., and Lewtas, J. DNA adducts in chronic degenerative diseases: pathogenetic relevance and implications in preventive medicine. *Mutat. Res.*, 366: 197-238, 1996.
34. Witschi, H., Espirito, I., Yu, M., and Willits, N. H. The effects of phenethyl isothiocyanate, *N*-acetylcysteine and green tea on tobacco smoke-induced lung tumors in strain A/J mice. *Carcinogenesis (Lond.)*, 19: 1789-1794, 1998.
35. Witschi, H., Uyeminami, D., Moran, D., and Espirito, I. Chemoprevention of tobacco-smoke lung carcinogenesis in mice after cessation of smoke exposure. *Carcinogenesis (Lond.)*, 21: 977-982, 2000.
36. Bryant, M. S., Skipper, P. L., Tannenbaum, S. R., and MacIure, M. Hemoglobin adducts of 4-aminobiphenyl in smokers and non-smokers. *Cancer Res.*, 47: 602-608, 1987.
37. Röster, S., Behr, J., and Richter, E. *N*-Acetylcysteine treatment lowers 4-aminobiphenyl haemoglobin adduct levels in non-smokers. *Eur. J. Cancer Prev.*, 8: 469-472, 1999.
38. Arif, J. M., Gairola, C. G., Glaser, H. P., Kelloff, G. J., Lubet, R. A., and Gupta, R. C. Effects of dietary supplementation of *N*-acetylcysteine on cigarette smoke-related DNA adducts in rat tissues. *Int. J. Oncol.*, 11: 1227-1233, 1997.
39. Arif, J. M., Gairola, C. G., Glaser, H. P., Kelloff, G. J., Lubet, R. A., and Gupta, R. C. Inhibition of cigarette smoke-related lipophilic DNA adducts in rat tissues by dietary Oltipraz. *Carcinogenesis (Lond.)*, 19: 1515-1517, 1998.
40. van Zandwijk, N., Moas, L. M., Breedijk, A., Godschalk, R. L. W., Salud, M., Wighout, G., Baas, P., van't Veer, L., van Aken, B., De Flora, S., and van Schooten, F. J. Aromatic DNA adduct levels in alveolar macrophages and lymphocytes of smoking volunteers: effects of *N*-acetylcysteine (NAC) supplementation (Abstract). In: Eighth World Conference on Lung Cancer, Dublin, Ireland, August 10-15, 1997.
41. De Flora, S., Camoirano, A., Bagnasco, M., Bennicelli, C., van Zandwijk, N., Wighout, G., Qian, G.-s., Zhu, Y.-r., and Kensler, T. W. Smokers and urinary genotoxins: implications for selection of cohorts and modulation of endpoints in chemoprevention trials. *J. Cell Biochem.*, 25 (Suppl.): 92-98, 1996.
42. Li, Z. Q., Luo, X. M., Wei, H. J., Feng, Y. M., and Zhang, C. Inhibition of the transforming ability of cigarette smoking condensate-treated human fetal lung DNA induced by oltipraz. *J. Environ. Pathol. Toxicol. Oncol.*, 13: 133-135, 1994.
43. De Flora, S., Izzotti, A., Walsh, D., Degan, P., Pettrilli, G. L., and Lewtas, J. Molecular epidemiology of atherosclerosis. *FASEB J.*, 11: 1021-1031, 1997.